Plant Endoplasmic Reticulum-Plasma Membrane Contact Sites (EPCS)

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Abstract

The endoplasmic reticulum (ER) acts as a superhighway with multiple side roads that connects the different membrane compartments including the ER to the plasma membrane (PM). ER—PM contact sites (EPCS) are a common feature in eukaryotic organisms, but have not been studied well in plants, due to the lack of molecular markers, and the difficulty in resolving the EPCS structure using conventional microscopy. Recently however, plant protein complexes required for linking the ER and PM have been identified. This is a further step towards understanding the structure and function of the plant EPCS. Here, we will highlight some recent studies in this field and suggest a number of hypotheses that relate to the possible function of EPCS in plants.

Plant EPCS and the ER network

The cortical endoplasmic reticulum (ER) in plants forms a dynamic geometrical network of tubules and small cisternae underlying the plasma membrane [1]. The dynamics of this network and to a certain extent its geometrical organisation are in part controlled by the cortical actin network [2] and a number of members of the myosin XI family [3, 4]. However, depolymerisation of the cortical actin by drugs such as cytochalasin B and latrunculin B, although perturbing the ER network, do not result in its destruction [5]. The implication being that perhaps ER tubules are anchored to other cellular components such as the microtubule cytoskeleton, or the plasma membrane (PM).

Cortical ER links with the PM through ER—PM contact sites

Early electron microscopy studies identified sites of contact between the ER and PM; the so called ER—PM contact sites (EPCS) [6]. More advanced light microscopy technologies using persistency mapping of ER tubule dynamics further demonstrated the existence of the EPCS [7, 8]. Although their structure and function in plants remains to be fully evaluated, here we propose some possible models for the function of the EPCS that relate to their interesting structures (Key Figure 1, A). Morphologically, EPCS can be defined as a region where the two membranes are closely attached (less than 10 nm apart) and where ribosomes are excluded (Key Figure 1, B). In mature plant cells, the central vacuole occupies most of the volume. As

a result, the cytoplasm is restricted to a thin layer at the cell cortex and organelles such as the Golgi bodies and cortical endoplasmic reticulum reside very close to the PM [9].

The dynamic properties of the plant ER network are regulated by the cytoskeleton [10-12]; and the most obvious role of the EPCS would be to anchor the fast remodelling network. However, the physiological functions of EPCS are most likely to be far more complex. Studies conducted in yeast (*Saccharomyces cerevisiae*) have revealed that EPCS are sites of phospholipid metabolism and signalling [13, 14]. For example, the EPCS localised oxysterol homology-binding proteins (Osh) sense the level of PI4P at the PM, and facilitate the interaction with an integral ER protein, Scs2p, and Sac1 (a PI phosphatase). These interactions close the gap between cortical ER and the PM, allowing ER localised Sac1 to convert PI4P to PI at the PM [13]. In addition, Scs2p (known as VAP or VAP27 in animals and plants), is also required for multiple lipid synthesis and transport pathways [15, 16]. Studies in animal cells revealed that calcium transport from the extracellular space is regulated through a protein complex (STIM1 and Orai1) localised at the ER—PM junctions [17-21], and such protein complexes are also associated with actin bundles at the leading edge of migrating cancer cells [22].

Proteins identified in the EPCS complex in plants

A number of other proteins that localize to the EPCS have been identified and their molecular function in yeast and animals is reasonably well established [18, 19, 23]. However, their plant counterparts have not been well characterised (Table 1). Recently, a protein complex forming an EPCS in plants has been identified. This complex is composed of VAP27, NET3C, microtubules and actin filaments [24, 25]. NET3C belongs to the NETWORKED super-family of actin binding proteins [26, 27]. Members of this family form links between actin filaments and different membrane systems and they are specific to higher plants. Using either VAP27 or NET3C as markers, further studies have revealed more components of the EPCS in plants, such as synaptotagmin 1 that also confers mechano-tolerance (Key Key Figure 1, C-E)[28,29]. Proteins of the EPCS could be spatially closely associated or exactly co-locate at the same site (Key Figure 1,F-G) [30]. In this opinion article we evaluate this step forward in our understanding of the biological relevance of the plant EPCS and

hypothesize on their possible functions which could include regulating membrane trafficking pathways, cargo transport, calcium signalling and responses to biotic stress (Key Figure 1A).

The formation of EPCS in different cell types and roles in lipid transport

In our studies, when VAP27-GFP was stably expressed in arabidopsis (*Arabidopsis thaliana*) under its endogenous promoter, the formation of VAP27 labelled EPCS largely depended on the cell type [25]. This may reflect different requirements of EPCS in these cell types, such as in their ER remodelling capacity.

The function of plant EPCS in lipid transfer is poorly understood. There is evidence to suggest that the transport of oxysterol in plants is regulated by an interaction between VAP27 proteins and the ORP (oxysterol-binding-related protein) family by a mechanism that has been described in yeast [15]. There are 12 ORP genes identified in the arabidopsis genome, and they have different intracellular locations [31]. The ER/Golgi localised ORP3a binds to sterol *in vitro* [32] and a similar activity can be predicted for other ORP proteins that contain oxysterol binding domains. The petunia version of ORP1 localises to distinct foci that associate with the PM of pollen tubes [31], and this localisation is reminiscent of that of the EPCS. We propose that oxysterol trafficking in plants is likely to be mediated by the ORP-VAP27 complex, and such events could occur at the EPCS. In addition, the export of lipids that form protective structures, such as waxes and the cuticle, may also occur at the EPCS. The cuticle contains very-long chain fatty acids that are generated in the ER and transported through the PM localized ABC transporter to the extracellular space. This process is independent of the conventional Golgi dependent pathway, and is likely to be involved in the direct communication of the ER and PM localised transporters [33]. However, no clear evidence for EPCS mediated lipid transfer has yet been reported in plants. Future studies on determining the localization of various lipid transporter/binding proteins in relation to EPCS complex could provide valuable information in this aspect.

EPCS acts as the hub for the organization of the cytoskeleton

ER movement is regulated by the cytoskeleton and both actin filaments and microtubules are found closely associated at the site where ER is anchored to the PM [24]. In fission yeast,

ER—PM contact sites regulate the formation of the actomyosin contractile ring, which is made from dense actin filaments and is required for cell division [34]. In plants, a large number of EPCS overlap with the site where cortical microtubules and actin intersect (Figure 2, A-C) [24, 25, 28].

NET3C is the best example of an actin binding protein that has been shown to be part of the EPCS complex in plants, where it acts as an adapter between the actin cytoskeleton and the PM. Other potential candidates for EPCS actin binding proteins are myosin VIlls. A myosin VIII tail domain deletion mutant of ATM1 localises to the PM as well as numerous stationary puncta that sit closely to the three-way junctions of ER membrane [35]. This location is reminiscent of known EPCS proteins. Further evidence is required to confirm this but it is likely that actin-motor proteins participate in the EPCS where cargo transport and membrane trafficking may occur [36]. Another potential candidate for an actin binding EPCS component is FORMIN. For example, FORMIN 1 spans the PM [37], interacting with the cytoskeleton on the cytoplasmic domain and is in contact with the cell wall through its extracellular domain which constrains the movement of the protein in the PM [38]. A similar constraint is observed for VAP27 at the EPCS and this is likely to be due to some indirect binding involving a PM integral membrane protein [25]. Moreover, FORMINS are able to interact with actin filaments and microtubules [39, 40], and this property makes them ideal candidates for keeping the EPCS complex stationary as the dual association of VAP27 with microtubules and the actin cytoskeleton has also been reported (Figure 3, A) [24, 25].

The SCAR complex regulates ARP2/3 mediated actin filament nucleation [41]. Previous studies have shown that some SCAR complex components, are either PM or ER associated [42-45]. Proteins from the SCAR complex (e.g. NAP1) and the Arp2/3 complex (ARP3) localise to distinct puncta that associate with the ER surface as well as the cytoskeleton [45, 46]. Some of these NAP1 labelled puncta also overlap with EPCS that are labelled with VAP27-1 [45]. Taken together, these data suggest that functional ARP2/3 complexes or actin regulatory proteins can be present at the EPCS, facilitating actin cytoskeleton dynamics and remodelling.

Could EPCS be involved in pathogen and symbiont responses?

Dramatic cytoskeletal rearrangements and membrane/organelle movement occur in response to infection and symbioses [47-49]; dense ER membrane and F-actin cables are normally found at the site where the pathogen touches the cell surface [50]. On the other hand, such ER—PM associations have also been seen during symbioses, such as during infection thread formation in root hairs and during nitrogen fixation and the formation of mycorrhizal arbuscules [51]. Plasma membrane resident proteins localise to oomycete haustoria, and EPCS resident proteins such as synaptotagmin 1 can also be found concentrated at these sites (Figure 3, B) [52]. Therefore, could the EPCS function as hubs that react to extracellular signals such as pathogen infection? This is an intriguing hypothesis, which is supported by three lines of evidence: (i) Plant EPCS complexes have the capacity to recruit cytoskeletal associated proteins and this function might be elevated during infection where enhanced actin-membrane activity is required. (ii) Plant EPCS associate indirectly with the cell wall (Figure 3, C) [25], and might be able to react to certain extracellular signals. (iii) VAP27-1 interacts with a disease resistant protein (fungal pathogen protein), Cf-9, that forms membrane associated complexes [53, 54]; it also interacts with a hypersensitive response gene, ACD11 [55], all of which are required for the activation of plant defence during fungal infection. Thus, future studies should address the behaviour of EPCS complexes during infection and whether the susceptibility of plants changes when the function of the EPCS complex is compromised.

ER—PM contact sites and cell-cell communication

Plasmodesmata (PD) are a unique feature of plant cells forming 50nm diameter channels between cells. They are sites where the PM, ER and the actin cytoskeleton are known to converge [56]. Therefore, PD can be regarded as specialised ER—PM junctions. However, the number of EPCS on plasma membranes is greater than the number of PDs, especially at the cortex of epidermal cells, which obviously do not have plasmodesmata in their outer periclinal walls. At cell-cell borders, a large number of EPCS are found co-localised with PD, suggesting that EPCS acts as a scaffold that supports the central desmotubules through the PD [25]. More interestingly, recent studies also demonstrate that certain members of a family of ER curvature inducing proteins, the reticulons, are found in the developing PD and

may be required for constricting desmotubules [57]. A proteomics screen, supported by *in vivo* fluorescence resonance energy transfer experiments revealed that two of the PD localised reticulons (RTN3 and RTN6) interact with the EPCS proteins, VAP27 and synaptotagmins [58].

Plasmodesmata mediate the cell-to-cell transport of macromolecules as well as virus particles. Virus movement proteins (MP) localise to mobile punctate structures containing RNA, and these particles are regarded as the viral replication complexes (VRCs). These VRCs move on the cortical ER network, and they transiently pause at the cortical microtubule-associated ER sites (C-MERs) [59], which are structurally very similar to the EPCS. Therefore, viruses may use EPCS (or C-MERs) as a centre for the recruitment of membranes/proteins (transported through the cytoskeleton) that are required for their replication. When the formation of virus particles is complete, they can potentially be re-located using MPs to the PDs that are adjacent to EPCS and therefore ready to infect neighbouring cells. This hypothesis is supported from a few protein—protein interaction studies which suggest that the EPCS protein, VAP27-1, is involved in the viral replicative cycle, and interacts with virus proteins [60, 61]. In addition, a study using the EPCS localized synaptotagmin 1 and *Turnip vein clearing virus* suggests that synaptotagmin 1 interacts with MPs and mediates the trafficking of VRCs to the PD [29, 62].

Could plant EPCS provide the route a junction for membrane trafficking?

In plants, the Golgi and ER network are closely associated [1, 5]. Although the movement of Golgi bodies is not required for cargo sorting and trafficking activities, Golgi stacks often pause in association with stable ER junction associated microtubules, the cortical microtubule-associated ER sites (C-MERs), which are structurally similar to the EPCS [59, 63] (Figure 3, D). Thus, it could be postulated that such interactions are involved in transporting specific cargos, such as delivering cellulose synthase proteins whose secretion require pausing of Golgi bodies on the microtubules [64]. Alternatively EPCS could simply form a structural barrier which Golgi bodies need to overcome during their transit around the ER network.

EPCS localised proteins are also believed to regulate the endocytic pathway [62]. Studies using arabidopsis SYT1 demonstrate that it co-localises with FM4-64 labelled endocytic membranes, and over-expressing a dominant negative version of SYT1 inhibits the formation of plasma membrane-derived endosomes [62]. In mammalian, fungal and plant cells, endosomes are found closely associated with the ER network [65, 66]. Changes in the ER network structure and homeostasis (by changing the expression of ER structural proteins, e.g. reticulons, or RHD3) is critical for plant endosome streaming and endocytosis [65]. Direct interaction between endosome membrane proteins (e.g. StAR related lipid transfer domain-3, STARD3) and VAP proteins has been observed in animal cells [67], and interaction between VAP, retromer subunits and WASH proteins (ARP2/3 complex activators of actin nucleation) controls the formation of endosomes [68]. A similar phenomenon might exist in plants. For example, endocytotic vesicles form at the ER—PM site enriched in cytoskeletal components where the actin cytoskeleton provides the force for endocytotic vesicle formation and the closely associated ER membrane provides the track for endosome movement and distribution (Figure 3, G).

Concluding remarks

The organelles and membranes in a cell are not structurally isolated. More and more evidence supports the phenomenon that interactions and connections are found amongst membrane bounded organelles and the ER network [69-71]. The ER—PM connection, a conserved link observed across phylogeny, is one of the best known structures in this field. Proteins involved in this ER—PM connection in plants have recently been discovered. Based on the evidence we have to-date, we suggest that the plant EPCS are involved in maintaining the ER network structure, involved in lipid transport and phospholipid signalling using a pathway and molecular machinery that is similar to that in yeast and animal cells. Because of the association with the cytoskeleton and possible involvement of actin motor/regulator proteins, the EPCS in plant cells could play a central role in regulating cortical membrane-cytoskeleton dynamics. Consequently, they may act as a hub that senses various extracellular stimuli and signal [72], recruits various components that are required for the response to pathogen infection and for viral movement and replication. Hopefully,

these hypotheses will be tested in the near future and the biological relevance of EPCS, including their influence on membrane trafficking, cytoskeletal function, plant growth and development will become clearer.

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Figure legends

Table 1. Known ER—PM contact site proteins in yeast, animals, and plants.

Knownn ER—PM proteins in different species			Notes	Refs
Yeast	Animal	Plant		
n/a	STIM1/Orai1	n/a	ER localised STIM1 interact with a PM calcium channel, Orai1, regulating calcium influx	[17,19,20,22]
n/a	STIMATE/TMEM110	n/a	Interact with STIM1 and regulate calcium signalling	[18,21]
Scs2,Scs22	VAP-A,VAP-B	VAP27-1,VAP27-3	Interact with various lipid interacting proteins and mediate lipid transport	[14,16,24,25]
Osh	ORP	ORP	It binds to oxysterol., and also regulates PI4P metabolism	[13,15,31,32]
Tcb1-3	E-Syt	Syt1	It brings ER close to the PM in response to Calcium signal	[23,28-30,62]
Ist2p	n/a	n/a	Cortical ER protein involved in ER—PM tethering, and regulates cellular ion homeostasis	[23]
n/a	n/a	NET3C	Plant specific NET family, and interact with actin cytoskeleton	[24-27]

Key Figure 1. Summary of possible functions of the plant ER—PM contact sites in plants, and the localization of known EPCS candidates. (A) Multiple functions of plant EPCS are proposed. EPCS may be involved in various cellular processes, such as membrane trafficking. Specific cargos may be transported through EPCS via Golgi bodies (1), and EPCS could also provide a route for endocytosis (2). EPCS are also likely to be involved in regulating the calcium signalling pathway (3), controlling viral movement through EPCS-PD interactions (4) and mediating responses to extracellular stimuli such as biotic stresses (5). **(B)** Transmission electron micrograph depicting a 70nm section of an outer integument cell of a developing *Cardamine hirsuta* seed (fruit development stage 15) after high pressure freezing and freeze

substitution. Numerous ER-plasma membrane contact sites can be seen all around the cell circumference. (1, 2) High magnification images show that ER and plasma membrane are in close physical contact and that contact sites can stretch over several hundreds of nanometers. Note the absence of ribosomes on the ER surface facing the plasma membrane. Images courtesy of Ulla Neumann and Angela Hay, MPIPZ, Cologne. **(C-E)** Subcellular localization of selected EPCS proteins in plants is illustrated. These include VAP27-1 (B), NET3C (C), and synaptotagmin, all of which label persistent ER nodules known to be ER—PM contact sites [24, 25, 30]. (F-G) Images showing the co-localization of NET3C with SYT1 and VAP27-1 respectively; NET3C and VAP27 localize to the same sites, whereas there is only partial over-lap of NET3C and SYT1 [30] (Scale Bar = 10µm for confocal; 0.5 µm for TEM).

Figure 2. Plant EPCS associate with the cytoskeleton. (A-C) EPCS are found closely associated with the cytoskeleton. The localization of VAP27-1 labelled EPCS often follows the pattern of microtubules especially in mature trichome cells (A). Co-alignment between EPCS and the actin cytoskeleton is also significant (B), and a number of EPCS locate to the point where actin filaments and microtubules intersect (arrow, C) [26].

Figure 3. Suggested models and possible functions of plant EPCS. (A) Diagram illustrating the interaction of plant EPCS and the cytoskeleton. EPCS complexes interact with microtubules directly where they may recruit certain actin regulatory proteins and regulate actin organization. **(B)** EPCS localise to the oomycete haustrorium, as a consequence, dense ER and actin filaments are found at these sites. **(C)** ER/PM contact sites (VAP27-1-YFP, red) have been found within the hechtian strands (green) after plasmolysis, most of which were located at the tips of the strands indicating they are connected to the cell wall**. (D)** In plants, Golgi bodies can interact with EPCS transiently, and this process may be involved in transporting specific cargos. Furthermore, endocytosis could also occur around EPCS in plants. The EPCS associated actin filaments provide the force for PM invagination and the ER membrane provide the track for endosome movement (Scale Bar = 10µm).

Glossary

ER—PM contact sites (EPCS)

Also known as ER—PM junctions or ER—PM anchor sites, they are close appositions between the two structures. They exist in different species, and their main functions studied so far are lipid transport and calcium homeostasis.

VAP27 Protein

It is known as Scs2 in yeast and VAP in animal cells, all of which localise to EPCS. There are 10 homologues of VAP27 in arabidopsis, and all contain a Major Sperm Domain, which is believed to interact with proteins containing a FFAT motif.

Synaptotagmins (SYT)

They were first known as a Ca^{2+} sensor in the membrane of the pre-synaptic axon terminal in animals. They contain a C-terminal C2-domain, which binds to phospholipids in response to calcium signals. There are 5 homologues found in the arabidopsis genome, and SYT1 has been shown to localise to EPCS.

NET proteins

NETWORKED super-family of actin binding proteins, found specifically in plants. Members of this family form links between actin filaments and different membrane systems.

STIM1/Orai1

This protein complex is required for Ca^{2+} transport in animal cells. The ER localized STIM1 interacts with the PM Ca^{2+} channel , Orai1, when the Ca^{2+} levels in the ER is low. However, these proteins do not exist in plants.

SCAR and ARP2/3 complexes

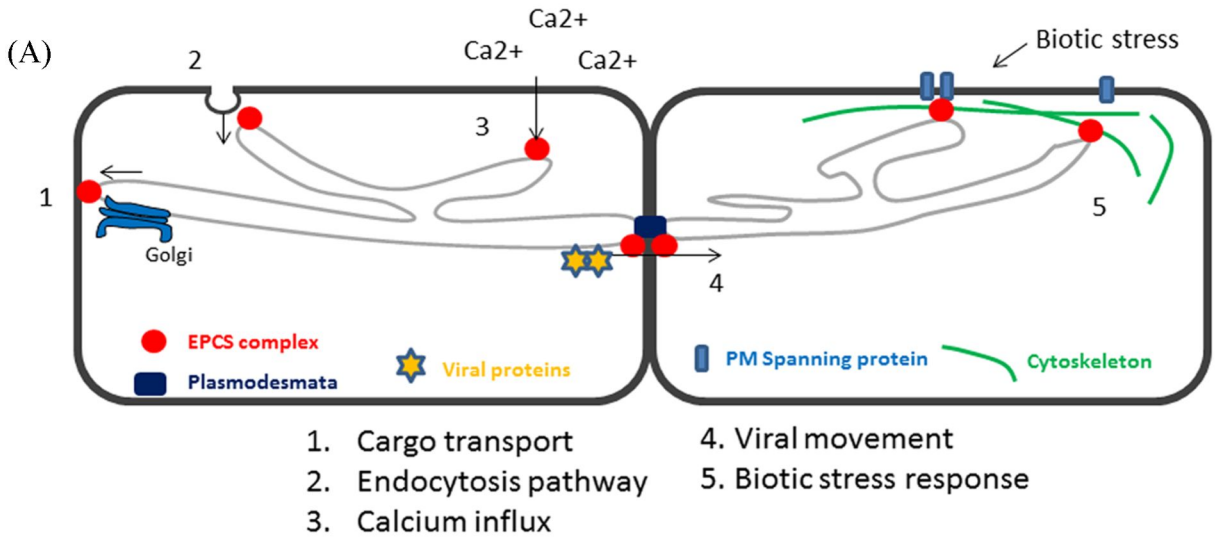
ARP2/3 complex mediates actin nucleation, and its activity is regulated by the SCAR complex that contains multiple proteins, such as NAP1 and PIR121. This machinery is conserved in most eukaryotes.

Plasmodesmata

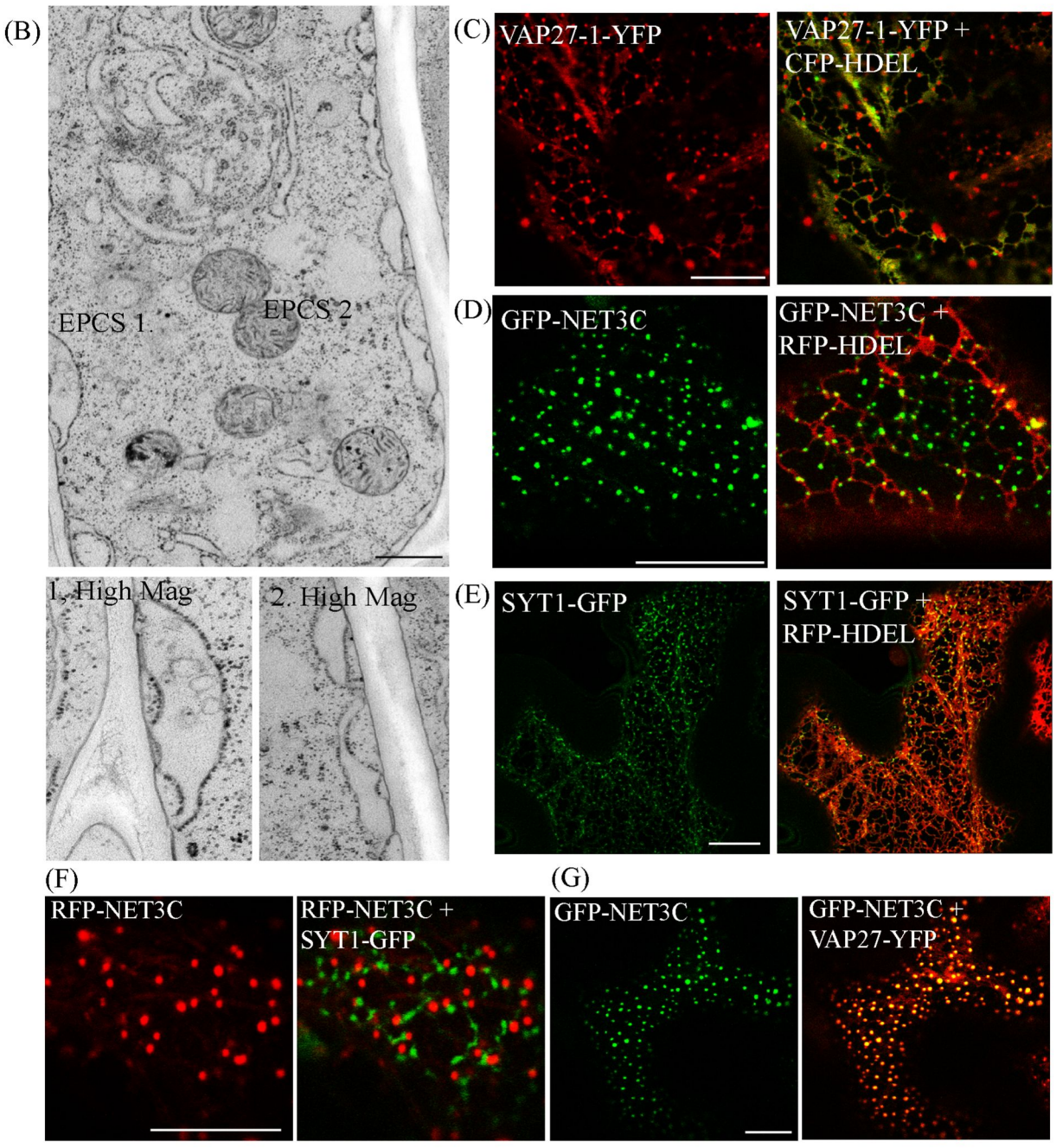
Plasmodesmata (PD) are a unique feature of plant cells forming channels that mediate cell-to-cell transport.

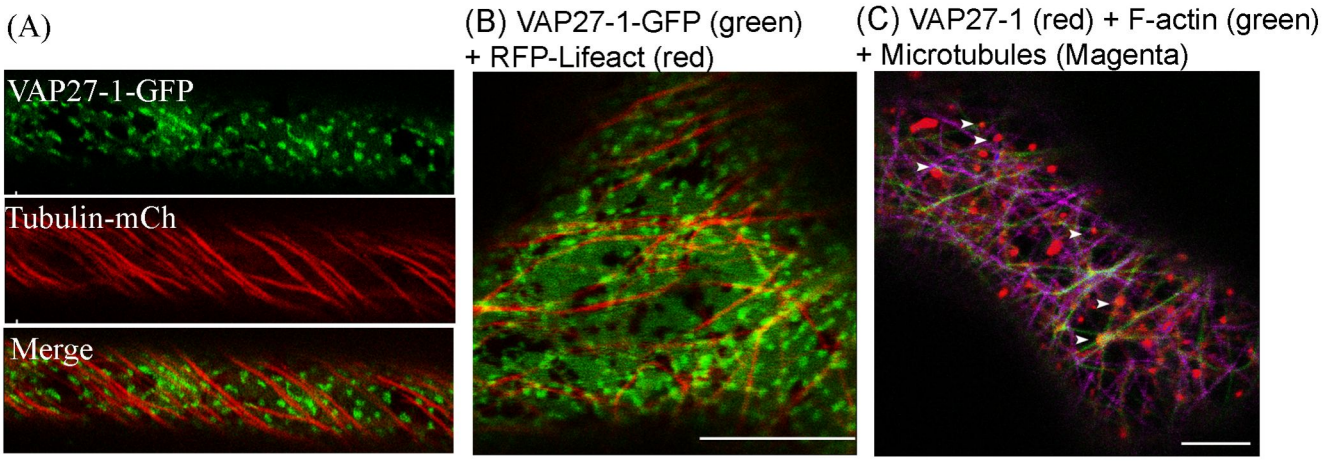
C-MERs

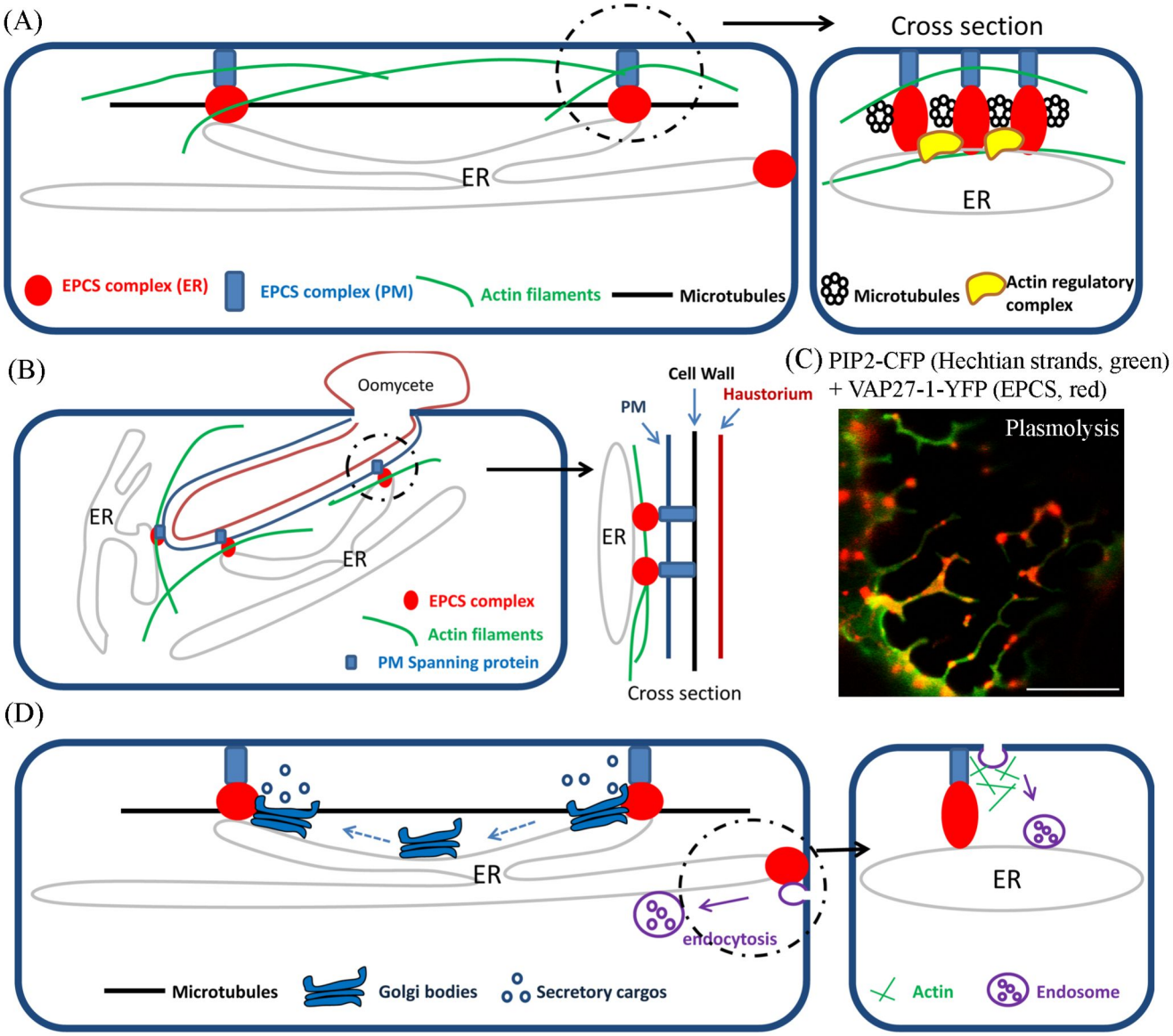
Cortical microtubule-associated ER sites, they intersect with the ER-actin network and mark the position of pausing organelles. Most of them are stable and structurally similar to EPCS. The association of microtubules at the C-MER places it adjacent to, but not directly on the EPCS.



1. Cargo transport
2. Endocytosis pathway
3. Calcium influx
4. Viral movement
5. Biotic stress response




(A)
VAP27-1-GFP
Tubulin-mCh
Merge
(B) VAP27-1-GFP (green)
+ RFP-Lifeact (red)
(C) VAP27-1 (red) + F-actin (green)
+ Microtubules (Magenta)


(A)
Cross section
ER
ER
EPCS complex (ER)
EPCS complex (PM)
Actin filaments
Microtubules
Microtubules
Actin regulatory complex
(B)
Oomycete
ER
ER
EPCS complex
Actin filaments
PM Spanning protein
Cell Wall
Haustorium
PM
ER
Cross section
(C) PIP2-CFP (Hechtian strands, green) + VAP27-1-YFP (EPCS, red)
Plasmolysis
(D)
ER
endocytosis
Microtubules
Golgi bodies
Secretory cargos
ER
Actin
Endosome